

**McConnell AT, Ellis R, Pathy B, Plummer R, Lovat PE, O'Boyle G. The prognostic significance and impact of the CXCR4/CXCR7/CXCL12 axis in primary cutaneous melanoma. *British Journal of Dermatology* 2016. DOI: 10.1111/bjd.14720**

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**DOI link to article:**

<http://dx.doi.org/10.1111/bjd.14720>

**Date deposited:**

31/05/2016

**Embargo release date:**

11 May 2017

The prognostic significance and impact of the CXCR4/CXCR7/CXCL12 axis in primary cutaneous melanoma

RUNNING TITLE: Significant impact of the CXCR4/CXCR7/CXCL12 axis in primary melanoma

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FUNDING: This work was support by the Medical Research Council, Cancer Research UK and The British Skin Foundation

CONFLICTS: The authors have no conflicts of interest to disclose.

### **What is already known about this topic?**

- Chemokine receptor 4 (CXCR4) expression is associated with poor prognostic indicators including tumour ulceration, thickness and metastasis.
- Expression of CXCR4 is crucial in regulating melanoma chemotaxis to distant secondary sites of metastasis with high expression of the ligand, CXCL12.
- Previous studies suggest a lack of correlation between melanoma CXCL12 expression and disease progression or BRAF mutational status
- CXCR7 is an atypical/decoy CXCL12 receptor which may act to shape CXCL12 gradients

### **What does this study add?**

- Elevated CXCR4 expression is a prognostic biomarker for AJCC stage II primary melanomas
- Autocrine melanoma CXCR4/CXCL12 signalling promotes hyper-activation of pro-survival MAPK signalling
- CXCL12 expression by stromal cells in the primary melanoma microenvironment protects from metastasis potentially by retaining tumour cells within the cutaneous microenvironment
- Downregulation of melanoma CXCL12 is associated with an aggressive BRAF/NRAS mutant phenotype
- CXCR7 expression by cutaneous vascular endothelium and not primary melanomas suggests a mechanism mediating tumour intravasation

### **What is the translational message?**

CXCR4 expression defines a potential candidate biomarker for melanoma providing additional prognostic information to AJCC disease staging and a means of identifying high risk patient sub groups. Additionally the reported role of CXCR4/CXCR7/CXCL12 chemokine signalling within the primary cutaneous melanoma microenvironment highlights the potential for targeting this chemokine axis to prevent the development of metastatic disease.

## ABSTRACT

### Background

Expression of the chemokine receptor CXCR4 is known to regulate melanoma metastasis to distant sites with high expression of CXCL12 ligand, but the prognostic impact of CXCR4 expression and potential for autocrine mediated activation of pro-survival mitogen-activated-protein-kinase signalling remains enigmatic. Furthermore expression of the decoy receptor CXCR7, within the local cutaneous melanoma microenvironment remains undefined.

### Objectives

The current study aimed to define the contribution and prognostic impact of CXCR4/CXCR7/CXCL12 signalling in primary cutaneous melanomas and immediate tumour microenvironment.

### Methods

Immunohistochemical/immunofluorescent expression of CXCR4, CXCR7 or CXCL12 was analysed in human metastatic melanoma cell lines, primary cutaneous cell types and in a retrospective cohort of primary melanomas/benign nevi. CXCL12 secretion by melanoma/cutaneous cells was evaluated by enzyme-linked Immuno-absorbent assay and autocrine CXCR4/CXCL12 signalling investigated by addition of a CXCL12 neutralising antibody.

### Results

CXCR4 expression was significantly higher in primary melanomas which subsequently metastasised after 7 years ( $P=0.037$ ). Stratification for AJCC stage II disease revealed significantly decreased disease free survival in patients with  $>50\%$  CXCR4 expression ( $P=0.036$ ) while comparative analysis of CXCL12 expression in the adjacent epidermis of all AJCC stage melanomas revealed increased CXCL12 correlated with prolonged time to metastasis ( $P=0.014$ ). CXCR7 expression was expressed within the primary melanoma microenvironment but was absent on primary tumours. Addition of anti-CXCL12 to BRAF mutant melanoma cells resulted in down regulation of phospho-CXCR4 and phospho-ERK, indicating autocrine CXCR4/CXCL12 signalling.

## Conclusions

CXCR4 expression defines a potential prognostic biomarker for AJCC stage II melanoma. Moreover, targeting the CXCR4/CXCR7/CXCL12 axis maybe represent a novel therapeutic strategy to prevent early melanoma progression.

## INTRODUCTION

Cutaneous melanoma is the most aggressive form of skin cancer, with an increasing worldwide incidence, particularly in younger individuals for which death from metastatic disease accounts for 80% of all skin cancer deaths. Moreover, despite recent advances in targeted therapies to oncogenic BRAF/NRAS-induced pro-survival mitogen-activated protein kinase (MAPK) signalling and novel immunotherapies, there are still no consistently beneficial treatments for cutaneous metastatic melanoma or reliable biomarkers able to predict disease progression for those patients diagnosed with seemingly low risk, early AJCC stage primary tumours<sup>1,2</sup>. An urgent and improved understanding of the primary tumour microenvironment is therefore required in order to identify credible prognostic biomarkers able to predict those primary melanomas at greatest risk of disease progression and to inform the development of more efficacious precision based therapeutic strategies to prevent tumour migration and metastasis.

Chemokines and their receptors play a diverse and critical role in normal cellular homeostasis. Principally the chemokine system induces the directional migration of chemokine receptor expressing cells down chemokine ligand gradients, with involvement in processes as early as embryogenesis and in normal immune responses<sup>3</sup>. Unsurprisingly, cancer cells may hijack chemokine axes, upregulate chemokine receptors or secrete chemokine ligands, directly impacting on the behaviour of the tumour cell itself, the tumour microenvironment and establishment of metastasis, for which deregulation of CXCR4/CXCR7/CXCL12 signalling is well described mechanism of tumour cell migration and metastasis<sup>4</sup>.

The gain of CXCR4 expression by melanoma cells not only permits migration towards CXCL12 at common metastatic sites, but may also promote tumour cell survival and proliferation through paracrine/autocrine mediated activation of MAPK cell signalling, highlighting a potential role for CXCR4–CXCL12 signalling in melanomagenesis<sup>5,6</sup>. Furthermore, elevated CXCR4 expression has been linked to melanoma ulceration, thickness and disease progression<sup>7-10</sup>, although to date CXCR4 expression as a prognostic biomarker remains ambivalent<sup>11</sup>. Likewise, the role of CXCL12 chemokine

ligand within primary cutaneous melanomas has largely been overlooked. Importantly CXCL12 functions to shape the primary tumour infiltrate, promoting angiogenesis by recruiting potent pro-angiogenic plasmacytoid dendritic cells or creating an immunosuppressive tumour microenvironment through recruitment of CD4<sup>+</sup> T regulatory cells <sup>12</sup>. The effect of CXCL12 within the local microenvironment of primary cutaneous melanoma however, remains unexplored, with only one study reporting an apparent correlation between the absence of tumoural expression and poor prognosis <sup>11</sup>.

To add complexity to CXCR4-CXCL12 signalling, recent studies have identified CXCR7 as a chemokine receptor that binds to CXCL12 with 10 fold greater affinity compared to CXCR4 <sup>13</sup>. However, despite its classical features as a chemokine receptor, in general, CXCR7 functions as a 'decoy' receptor that 'scavenges' and hence removes monomeric CXCL12 to dampen or inhibit CXCR4-CXCL12 signalling. CXCR7 also has the ability to heterodimerise with CXCR4, thereby potentially modulating downstream signalling including proliferation, survival and cell migration. Indeed the up regulation of CXCR7 has been shown to promote growth and migration of a number of tumour types <sup>14-16</sup>, however, to date there are no reports of CXCR7 expression or function in melanoma.

Although known to influence the metastasis of melanoma to distant sites, the role of CXCR4-CXCR7-CXCL12 signalling in the primary melanoma local environment and its influence on early tumour migration and invasion remains unclear <sup>5</sup>.

In the present study we report high levels of CXCR4 expression within primary melanomas as a novel prognostic biomarker, identifying high risk AJCC stage II tumours. We further identify and characterise CXCL12 and CXCR7 expression within primary melanomas and the cutaneous microenvironment highlighting a link between relative CXCL12 expression and BRAF/NRAS mutational status and associated autocrine signalling and a mechanism by which the CXCR4-CXCR7-CXCL12 axis promotes melanoma migration and metastasis.

## METHODS

### **Patient demographics**

Retrospective formalin-fixed paraffin-embedded (FFPE) tissue was obtained from a cohort of 77 samples comprising 13 benign melanocytic naevi and 64 primary American Joint Committee on Cancer (AJCC) stage I, II or III melanomas at the time of diagnosis and following excision at the Royal Victoria Infirmary, Newcastle-upon-Tyne between January 2003 and May 2005. Full ethical permission was obtained (Newcastle and North Tyneside research ethics committee (Ref; 08/H0906/95+5) and all aspects of the study were performed in accordance with the Declaration of Helsinki Principles. Data were correlated with clinical outcome after a minimum of seven years follow-up, allowing the correlation of CXCR4 expression in the primary tissue sample with the time to the development of metastatic disease (disease free survival; DFS). Disease recurrence was defined by the time to first radiological or tissue diagnosis of metastatic disease (nodal or systemic) from the point of initial primary tumour excision, or time to death from melanoma (melanoma specific mortality; MSM). Reporting of the data was performed in line with the REMARK guidelines for prognostic biomarkers <sup>17</sup>.

### **Growth and Maintenance of Melanoma Cell Lines and Primary Skin Cells**

Human metastatic melanoma cell lines CHL-1 (BRAF/NRAS wild-type), A375, WM-164, (both BRAF V600E mutated) WM1361 (NRAS mutant) and EA.hy926 endothelial cell lines were obtained from the ATCC (Manassas, VA, USA) and cultured as previously described <sup>18,19</sup>, with authenticity of melanoma cell lines confirmed by melan A expression analysis <sup>20</sup> and using custom TaqMan SNP genotyping assays (Applied Biosystems, Warrington, UK) to verify BRAF/NRAS mutational status <sup>21</sup>. Primary human epidermal keratinocytes, dermal fibroblasts and melanocytes were isolated from redundant foreskin <sup>22</sup>, breast or abdominal skin, and primary cultures expanded in human keratinocyte growth supplement EpiLife medium (Invitrogen, Paisley, UK), Dulbecco's Modified Eagles Medium (Lonza, Vervies, Belgium) or melanocyte 254 medium (Life Technologies, Paisley, UK) respectively. Full ethical permission for the isolation of primary cells was obtained as described above.



## **Immunohistochemistry/Immunofluorescence**

Immunohistochemistry for the expression of CXCR4 and CXCR7 expression in formalin fixed paraffin embedded (FFPE) sections of naevi, primary melanomas or control tonsil was carried out as previously described <sup>23</sup>. CXCR4 was detected with monoclonal anti-CXCR4 (R&D systems clone 44716, 2.5µg/ml with antigen retrieval performed using 0.1M Tris-HCl pH 7.6), or CXCR7 with monoclonal anti-CXCR7 (Chemocentryx, San Francisco, USA, clone 11GB, 5µg/ml with antigen retrieval performed using 0.1M Tris-HCl pH 9) overnight at 4°C as previously described <sup>24</sup>. Isotype matched negative control antibodies were included during all staining to demonstrate antibody specificity. Images acquired by light microscopy with an x20 objective (Zeiss Axio Imager, Carl Zeiss Microscopy, New York, USA).

Immunofluorescence for the detection of CXCR7 and CXCL12 was carried out on metastatic melanoma and EA.hy926 cell lines, primary melanocytes, keratinocytes or dermal fibroblasts seeded at 2x10<sup>5</sup> cells/well for 24 hours on glass coverslips prior to fixation in ice cold 4% paraformaldehyde and permeabilisation with 0.2% Triton X-100. Immunolabeling of cells was performed with monoclonal anti-CXCR7 (Chemocentryx, San Francisco, USA, clone 11GB, 10µg/ml) or polyclonal anti-CXCL12 (Abcam, Cambridge, UK, clone 9797, 1ug/ml) overnight at 4 °C and detected with Oregon green 488 conjugated to anti- mouse or anti-rabbit IgG (Life Technologies, Paisley,UK) and nuclei counterstained with DAPI (Thermoscientific, Illinois, USA).

Fluorescent double staining of primary FFPE melanoma sections with CXCL12 and melan A (control melanocyte marker) was performed as previously described <sup>5</sup>. Antigen retrieval was performed in 10 mM Sodium citrate (pH 6) for 1 min and primary CXCL12 detected with rabbit anti-CXCL12 (Abcam, Cambridge, UK, clone 9797, 1µg/ml) or for melan A expression with mouse anti-MelanA (Abcam, Cambridge, UK, clone 731, 0.4ug/ml). Primary antibody binding was detected with Alexa Fluor® 568 conjugated to anti rabbit IgG (Abcam Cambridge, UK, clone 175471) or Oregon green 488 conjugated to anti-mouse IgG (Life Technologies, Paisley, UK) and nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI, Thermoscientific, Illinois, USA, 1ug/ml). Tumour sections or stained cells were finally mounted onto glass coverslips with fluorescence mounting medium (Vector Labs, Burlingame,

USA), and images captured by confocal microscopy using x40 or x20 objectives (Leica TSC SP2 UV, Leica Microsystems GmbH, Heidelberg, Germany).

### **Quantification of Immunohistochemical CXCR4 and immunofluorescent CXCL12 expression**

Mean % immunohistochemical expression of CXCR4 positively stained cells was derived by the analysis of up to ten representative fields of vision using Leica QWin image analysis software (Leica Microsystems) as previously described <sup>23</sup>, while CXCL12 fluorescence per tumour cell (relative to melan A) or within the epidermis of melanoma tissue sections (relative to DAPI) was quantified above background fluorescence from 4 or 2 representative images per tumour respectively using ImageJ software.

### **Western blotting**

Preparation of whole-cell lysates and western blotting for Anti-CXCR4 (Abcam Cambridge, UK, clone 2074, 0.1µg/ml) or Anti-Phospho-CXCR4 (Abcam Cambridge, UK, clone 74012, 1:1000) or Phospho-ERK (Cell Signalling, Danvers, MA, USA, clone 43775, 1:5000) and GAPDH (loading control) (Cell Signalling, Danvers, MA, USA, clone 2118, 1:5000) was performed as described previously <sup>18</sup>.

### **Enzyme Linked Immuno-absorbent assay (ELISA) for CXCL12**

Human melanoma cell lines were seeded at a density of  $5 \times 10^4$  cells/well in a 6 well plates for 72 hours prior to the analysis of CXCL12 in cell supernatants using a commercially available ELISA assay according to the manufacturer's instructions (Human CXCL12/SDF-1 alpha Quantikine ELISA Kit, R&D systems, DSA00).

### **Treatment of Cells with CXCL12 Neutralising Antibody**

WM-164 melanoma cells were seeded at  $1.5 \times 10^5$  cells/well in 6 well tissue culture plates 24 hours prior to the addition of 1µg/ml anti-CXCL12 neutralising antibody (Abcam Cambridge, UK, clone 9797) or IgG isotype control antibody (Abcam Cambridge, UK, clone 171870) for 5-30 minutes before harvesting cell lysates for western blotting.

## Statistics

CXCR4 immunohistochemical data were expressed as median expression levels between groups. Independent group analysis between localised and metastatic disease (eventual AJCC stage I/II versus eventual AJCC stage III/IV disease) was determined by Mann-Whitney *U*. Difference between “high” and “low” CXCR4 expression levels for survival curve analysis was tested using the Wilcoxon Signed Rank Test. Univariate and subsequent Log-rank (Mantel-Cox) survival analysis was undertaken using R 2.15.0 (R Foundation for Statistical Computing). Mann-Whitney *U* was used for analysis of CXCL12 fluorescence between localised and metastatic disease (eventual AJCC stage I/II versus eventual AJCC stage III/IV disease) or BRAF mutated and Wild-Type melanomas, while Pearson Product Moment Correlation analysis was used to correlate mean epidermal CXCL12 fluorescence with time to metastasis or cytoplasmic CXCR4 expression. Relative densitometry of P-CXCR4:GAPDH or P-ERK:GAPDH was analysed by One-way ANOVA with Dunnetts post hoc correction. A *P* value <0.050 was considered significant throughout. All data were analysed using statistical software Prism 5 (Graph Pad, San Diego, CA, USA).

## RESULTS

### CXCR4 Expression is Prognostic Biomarker in Cutaneous Melanoma

To assess CXCR4 expression in the context of melanoma, CXCR4 expression levels were initially compared *in vitro* in a panel of BRAF/NRAS wild type or mutated cutaneous melanoma cell lines with expression in primary cutaneous melanocytes. Western blot analysis of total CXCR4 expression revealed variable but consistent expression of CXCR4 in primary melanocytes as well as in cutaneous CHL-1 (BRAF/NRAS wild-type), WM-164 (BRAF<sup>V600E</sup> mutated) and WM-1361 (NRAS mutated) metastatic melanoma cell lines (Figure 1a & b), but with enhanced expression observed in all melanoma cell lines compared to expression in normal melanocytes.

Next, to determine the potential for CXCR4 expression as a prognostic biomarker for melanoma *in vivo* and as a means of identifying high-risk tumours at diagnosis, CXCR4 expression levels were evaluated

in a cohort of primary melanomas of differing AJCC disease stage. Primary melanomas showed variable expression of both cytoplasmic and nuclear CXCR4 (Figure 1c). Comparison of total CXCR4 expression levels in melanomas which remained localized with expression in tumours which subsequently metastasised after 7 years follow up (eventual AJCC stages I/II vs. stages III/IV) revealed significantly higher median CXCR4 expression in the metastatic cohort (56.08% vs 37.48% in AJCC III/IV; Mann–Whitney,  $P=0.037$ ) (Figure 1d), thus highlighting an association between CXCR4 up regulation and the development of metastatic disease, and consistent with the reported role of CXCR4 in cancer cell migration <sup>5</sup>. A Wilcoxon signed-rank test confirmed a differential cut off point of 50% CXCR4 expression as appropriate for the performance of survival curve analysis where “high CXCR4” expression was defined as greater than 50%, and ‘low CXCR4’ expression as less than 50%. Univariate analysis of mean percentage total CXCR4 expression in all AJCC stage melanomas revealed a non-significant decrease in disease free survival from 65.5% for individuals expressing low CXCR4 (< 50%), to 47.6% in individuals expressing high CXCR4 (>50%) over seven years (Log-rank (Mantel-Cox) test HR = 1.99 (95% CI 0.81 – 4.91)  $P = 0.131$ , Figure 1e). However, stratification for AJCC stage II melanomas at diagnosis, revealed high CXCR4 expression (>50%) was associated with significantly decreased disease free survival (reduced to 30.77%, compared to 73.68% for individuals with low (<50%) CXCR4 expression) and who remained disease free over 7 years (Log-rank (Mantel-Cox) test HR = 3.24 (95% CI 1.08 – 9.73) \* $P = 0.036$ , Figure 1f). Collectively, these results highlight a 3-fold risk of disease reoccurrence in individuals with high total CXCR4 expression (>50%) (Figure 1f), further suggesting high CXCR4 expression as a prognostic biomarker for AJCC stage II melanomas.

#### **Expression and Secretion of CXCL12 in Cutaneous Melanoma Cell Lines and Primary Cutaneous Cells**

To assess the role of CXCL12 in the primary tumour environment of cutaneous melanoma, initial immunofluorescence studies for expression of CXCL12 were performed in melanoma cell lines, as well as primary cells known to be present within the local tumour microenvironment including primary melanocytes, keratinocytes and dermal fibroblasts.

Immunofluorescent staining for CXCL12 revealed variable cytoplasmic expression by all cell lines and primary cells, indicating that under normal and unstimulated cell culture conditions, CXCL12 is likely produced *in situ* (Figure 2a). To investigate CXCL12 secretion by differing cutaneous cell types, CXCL12 was quantified in cell culture supernatants derived from confluent cutaneous melanoma cell lines, primary melanocytes, keratinocytes and fibroblasts using a commercial ELISA assay (Figure 2b). Surprisingly, although all cells displayed cytoplasmic expression of CXCL12, only CHL-1 (BRAF/NRAS wild-type) and WM-164 (BRAF<sup>V600E</sup> mutated) melanoma cells and dermal fibroblasts secreted detectable levels of CXCL12 (Limit of detection 156pg/ml; Figure 2b).

To investigate the possibility of CXCR4-CXCL12 autocrine cell signalling and impact on MAPK cell signalling, a neutralising antibody to CXCL12 or isotype control antibody was added to WM-164 cells. Western blotting revealed a significant reduction of phospho-CXCR4 expression after treatment with CXCL12 neutralising antibody for 10 or 20 minutes (Figure 2c & d; one-way ANOVA with Dunnetts post hoc correction \*\*P= 0.001), accompanied by significant inhibition of MAPK cell signalling and a concomitant reduction in the expression of phospho-ERK (Figure 2c & e; one-way ANOVA with Dunnetts post hoc correction \*\*P= 0.003).

### **Downregulation of Melanoma CXCL12 is associated with an Aggressive BRAF/NRAS Mutant Phenotype**

To investigate further the impact of CXCL12 autocrine signalling in cutaneous melanomas *in situ* and to determine the expression of CXCL12 in the immediate tumour microenvironment, CXCL12 expression was evaluated in a cohort of 24 melanocytic naevi or primary melanomas of differing AJCC disease stage with double staining for melan-A, used as a melanocytic marker (Figure 3a).

In line with previous observations, CXCL12 expression was detected within the dermis, localised to dermal fibroblasts <sup>25</sup>, and interestingly was also strongly expressed by stromal cells within the epidermis adjacent to all primary melanomas (Figure 4a). In contrast, tumoural CXCL12 in all AJCC stage primary cutaneous melanomas co-localised with melan-A expression (Figure 3a).

Analysis of CXCL12 within primary melanomas revealed consistent expression of CXCL12 by tumour cells, albeit at low levels. Expression, however, did not vary significantly between localised or metastatic melanomas (Figure 3b; Mann Whitney U  $P=0.113$  ns). Remarkably, comparison of CXCL12 expression with NRAS/BRAF mutational status revealed significantly reduced CXCL12 expression in BRAF/NRAS mutant tumours compared to expression in wild-type tumours (Figure 3c; Students T test  $*P=0.032$ ), suggesting that the down-regulation of CXCL12 secretion maybe mediated by activation of MAPK signalling.

### **Increased of CXCL12 in Primary Cutaneous Melanoma Epidermis Correlates with Increased Time to Metastasis**

To explore CXCL12 expression within the epidermis of primary melanomas and any association with disease progression, CXCL12 fluorescence was also quantified in the peri-tumoural adjacent epidermis of all primary melanomas within the study cohort. Results revealed consistent expression of epidermal CXCL12, in particular by keratinocytes in the stratum corneum (Figure 4a) in the adjacent epidermis overlaying all primary melanomas (Figure 4b). Comparative analysis of epidermal CXCL12 expression in the adjacent epidermis of all AJCC stage melanomas with tumour progression, further revealed a significant correlation between increased CXCL12 expression and increased time to metastasis (Figure 4c; Pearsons correlation  $*P=0.013$ ), suggesting CXCL12 within the immediate tumour microenvironment limits disease metastasis. Furthermore, a significant correlation of adjacent epidermal CXCL12 expression with tumoural expression of cytoplasmic CXCR4 was also observed (Figure 4d; Pearson's correlation  $**P=0.009$ ), highlighting a possible relationship between CXCL12 expression in the microenvironment with chemokine receptor activation in the tumour and proposing a previously un-described intimate relationship between microenvironmental CXCL12, melanoma phenotype and clinical outcome.

### **Expression of CXCR7 in Cutaneous Melanoma and the Primary Tumour Microenvironment**

There is a paucity of studies exploring the role of CXCR7 in melanoma biology and within the immediate cutaneous microenvironment given its potential effect on to the CXCR4/CXCL12 axis. To

address the potential impact of CXCR7 on this chemokine-receptor axis, immunofluorescent analysis for the expression of CXCR7 was performed in CHL-1 or WM-164 melanoma cell lines, or in primary melanocytes. Expression levels were compared with known CXCR7 expression levels in control EA.hy926 endothelial cells (Figure 5a). However, while cytoplasmic CXCR7 was clearly expressed by EA.hy926 cells, there was no apparent expression of CXCR7 by either cutaneous melanoma cell lines or by primary melanocytes (Figure 5a). To extend these *in vitro* findings, CXCR7 expression was determined in the study patient cohort where results reflected the observed *in vitro* expression pattern; no apparent expression of CXCR7 was evident in any primary melanoma or benign nevus. Intriguingly, however, expression of CXCR7 was clearly detectable within the tumour microenvironment of the dermis of seven primary melanomas; specifically detected on cells lining the lumen of blood vessels and capillaries (Figure 5b). However, these observations did not correlate with either AJCC disease stage or with BRAF/NRAS mutational status (data not shown). Collectively these data suggest that CXCR7 expression by the cutaneous vascular endothelium may act as a decoy receptor for CXCL12, modulating chemokines gradients within the tumour microenvironment.

## DISCUSSION

Tumour thickness and ulceration are two components of the AJCC melanoma staging system that currently provide the most important prognostic information for patients diagnosed with primary cutaneous melanomas<sup>26</sup>. However, neither these nor any other prognostic indicators are able to reliably identify 'high risk' patients, whose tumours will eventually progress, emphasising the acute demand for credible, novel biomarkers with predictive accuracy of metastatic progression.

Current evidence suggests chemokines and their receptors play a critical role in many stages of cancer progression with the CXCR4/CXCL12 axis known to be key to melanoma metastasis to distant secondary sites<sup>4</sup>. However, despite reports demonstrating an association of CXCR4 expression with adverse clinical features<sup>7-10</sup>, to date, no studies have characterised the prognostic significance of CXCR4 expression in well powered melanoma cohorts. In contrast to previous studies, reporting an

apparent correlation of CXCR4 expression with melanoma ulceration, increased thickness, metastasis and morbidity<sup>7-10</sup>, Mitchell *et al* suggest CXCR4 expression correlates with better prognostic features, such as the absence of mitosis or ulceration, tumour regression, and early AJCC stage disease<sup>11</sup>. Data from the current study, however, clearly demonstrate that elevated CXCR4 expression in melanoma significantly correlates with a metastatic phenotype. Consistent with these findings, individuals with AJCC stage II primary cutaneous melanomas with high CXCR4 expression (>50%) were 3 fold more likely to develop metastasis with concurrent reduced disease free survival. Collectively these data define CXCR4 expression in primary melanomas as a novel prognostic biomarker for AJCC stage II disease, thus identifying a high risk sub-group and enabling earlier therapeutic intervention.

Our data further show the expression of CXCL12 by melanoma cells and parenchymal cells within the cutaneous microenvironment and additionally define an autocrine signalling mechanism whereby tumoural secretion of CXCL12 is able to promote activation of pro-survival MAPK cell signalling. Indeed, previous studies have illustrated CXCL12-induced activation of MAPK p44/42 (ERK1/2) and p38<sup>27</sup>, and in the context of our data supports a role for endogenous CXCL12 driving MAPK cell signalling in melanoma. To extend these findings *in situ*, our data further demonstrated there was no significant difference in CXCL12 expression between melanomas which remained localised and those which subsequently metastasised. These results are corroborated by a previous study also revealing a lack of any significant correlation between CXCL12 expression and metastasis or morbidity<sup>10</sup>. This suggests a model whereby abundant endogenous CXCL12 within the primary site would act to retain CXCR4 positive tumour cells *in situ*, limiting their migration towards CXCL12 rich distant sites. Furthermore, CXCL12 expression by primary melanomas provides supporting evidence for autocrine CXCR4/CXCL12 cell signalling, which as shown in our *in vitro* studies, promotes MAPK cell signalling, the likely consequence of which is sustained tumour maintenance and survival.

Previous studies have not provided evidence for any association of CXCL12 expression in melanoma with NRAS/BRAF mutational status<sup>11</sup>. Importantly, our data demonstrate significantly less CXCL12 expression in BRAF/NRAS mutant melanomas compared to wild-type tumours. This indicates, for the



first time, an exciting potential mechanism for oncogenic BRAF/NRAS signalling in melanomagenesis, whereby oncogenic BRAF/NRAS signalling reduces CXCL12 expression by primary tumour cells. Similarly, previous studies suggest the absence of CXCL12 expression correlates with poor clinical outcome <sup>11</sup>. Collectively, these and our data thus propose the absence/downregulation of CXCL12 expression by BRAF/NRAS primary melanomas supports the emergence of an aggressive disease phenotype and the enhanced ability for CXCR4-positive tumour cells to metastasise.

To date, the presence of CXCL12 in skin is documented <sup>11</sup>, however the role of CXCL12 within the tumour microenvironment and its effect on the primary tumour has not been defined. Quantification of CXCL12 expression within the epidermis revealed a positive correlation with increased microenvironmental CXCL12 and increased time to metastasis. Similarly, increased tumoural cytoplasmic CXCR4 expression correlated with increased CXCL12 expression. These data are coherent since they suggest tumoural CXCR4 is chronically activated by microenvironmental CXCL12 and therefore internalised from the melanoma cell membrane. Importantly, chronic CXCR4 signalling by melanoma is supported by our *in vitro* observation of basal pCXCR4 levels; phosphorylation of CXCR4 being characterised by ligand occupancy and a pre-requisite of receptor internalisation <sup>28</sup>. Moreover, our data suggest that a high level of CXCL12 within the epidermis adjacent to primary melanomas may act to retain CXCR4 positive tumour cells *in situ*, thereby promoting radial growth and delaying melanoma vertical invasion into the dermis and subsequent metastasis to distant sites.

CXCL12 interacts with its signalling receptor, CXCR4, but also the atypical/scavenging receptor; CXCR7, recently re-designated as ACKR3 <sup>29</sup>. CXCR7 has a powerful capacity to scavenge CXCL12, and studies suggest serum CXCL12 levels may be increased 5-fold when CXCR7 is absent or inhibited <sup>30</sup>. Although expressed at a low levels within the primary cutaneous melanoma microenvironment, the observed expression of CXCR7 expression by vascular endothelial cells supports previous observations of CXCR7 expression by endothelial cells in breast and lung tumour associated vasculature <sup>31</sup> and the likelihood of CXCL12 sequestration, potentially altering chemokine gradients within the parenchyma and facilitating melanoma intravasation. Furthermore, although we did not observe any association

between micro environmental CXCR7 expression and melanoma progression, other studies suggest high CXCR7 expression is often associated with periods of accelerated tumour growth, hypoxia and angiogenesis<sup>32</sup>.

Overall, our data highlight a previously un-described role and cross talk of the CXCR4/CXCR7/CXCL12 chemokine axis within the primary cutaneous melanoma microenvironment and its intimate relationship with disease dissemination and clinical outcome. Moreover, the definition of high CXCR4 expression as a potential novel prognostic biomarker for AJCC stage II melanomas will enable earlier therapeutic intervention and improved patient outcome.

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## FIGURE LEGENDS

### Figure 1. CXCR4 Expression is Prognostic Biomarker in Cutaneous Melanoma

(a) Representative Western blot of CXCR4 and GAPDH loading control expression in CHL-1 (wild type) WM-164 (BRAF V600E mutated), or WM-1361 (NRAS mutated) human metastatic cutaneous melanoma cell lines or primary melanocytes (N = 4).

(b) Densitometric analysis of CXCR4 relative to GAPDH expression. Each bar represents the mean of 4 replicates of CXCR4 band intensity normalised to GAPDH band intensity (CXCR4/GAPDH) for each cell line or primary melanocytes derived from 4 independent donors, and expressed relative to the mean of each individual experiment (mean  $\pm$  SD, N = 4).

(c) Representative immunohistochemical expression of CXCR4 in a primary AJCC stage III cutaneous melanoma depicting both nuclear and cytoplasmic CXCR4 expression. Images were acquired by light microscopy with a magnification 20x scale bar = 50 $\mu$ m.

(d) Scatter graph representing the mean % positive CXCR4 staining cells in localised (eventual AJCC stage of disease I/II) and metastatic melanomas (eventual AJCC stage III/IV) after 7 years follow up. Horizontal bars represent median staining percentage (Mann-Whitney U \*P= 0.037).

(e) Univariate analysis of mean % CXCR4 expression and disease free survival (7 years) in all AJCC stage melanomas. Vertical lines represent individual patients developing a metastasis. Log-rank (Mantel-Cox) test HR = 1.99 (95% CI 0.81 – 4.91) P= 0.131 ns.

(f) Univariate analysis of % CXCR4 expression and disease free survival (7 years) in AJCC stage II melanomas. A significantly increased risk of metastasis was detected in AJCC stage II tumours expressing >50% mean CXCR4 compared to tumours expressing <50% mean CXCR4. Vertical lines represent individual patients developing a metastasis. Log-rank (Mantel-Cox) test HR = 3.24 (95% CI 1.08 – 9.73) \*P = 0.036.

**Figure 2. Expression and Secretion of CXCL12 in Cutaneous Melanoma Cell Lines and Primary Cutaneous Cells**

(a) Representative image from 3 replicate experiments for the immunofluorescent expression of CXCL12 in cutaneous metastatic melanoma cell lines (CHL-1, A375, WM-164), primary melanocytes, keratinocytes or dermal fibroblasts. Images were acquired with negative antibody control set to no detectable fluorescence. Green depicts CXCL12 positivity and blue DAPI nuclear staining. Images acquired by confocal microscopy with a magnification 40x scale bar = 20µm.

(b) CXCL12 (pg/ml) secretion by human metastatic cutaneous melanoma cell lines (CHL-1, WM-164, A375, WM-1361) and primary melanocytes, keratinocytes or dermal fibroblasts. Each bar represents mean CXCL12 secretion of 3 independent experiments of cell lines or from primary cutaneous cells derived from 4 independent donors +/- SD.

(c) Representative Western blot of Phospho-CXCR4 (P-CXCR4), Phospho-ERK (P-ERK), total ERK and GAPDH loading control expression in WM-164 (BRAF<sup>V600E</sup> mutated) metastatic melanoma cells treated with Anti-CXCL12 neutralising antibody or IgG isotype control for 5, 10, 20, 30 minutes. N=3.

(d) Densitometric analysis of P-CXCR4 relative to GAPDH expression. Each bar represents the mean of 3 replicates of P-CXCR4 band intensity normalised to GAPDH band intensity (P-CXCR4/GAPDH), and expressed relative to the mean of each individual experiment (mean ± SD, N = 3). Statistics acquired by one-way ANOVA with Dunnett's post hoc correction \*\*P= 0.001.

(e) Densitometric analysis of P-ERK relative to GAPDH expression. Each bar represents the mean of 3 replicates of P-ERK band intensity normalised to GAPDH band intensity (P-ERK/GAPDH), and expressed relative to the mean of each individual experiment (mean ± SD, N = 3). Statistics acquired by one-way ANOVA with Dunnett's post hoc correction \*\*P= 0.003.

**Figure 3. Downregulation of Melanoma CXCL12 is associated with an Aggressive BRAF/NRAS Mutant Phenotype**

(a) Representative image of Melan-A (green), CXCL12 (red) expression or nuclear DAPI (blue) staining in a primary AJCC stage III melanoma, including co-localisation plot of Melan-A and CXCL12 expression. Images were acquired with null primary control set to no detectable fluorescence for each channel and by confocal microscopy with a magnification 20x, scale bar = 150µm.

(b) Scatter graph representing mean of four high powered fields (4 HPF) of CXCL12 fluorescence/expression per melanoma cell in primary localised (eventual AJCC stage of disease I/II) or metastatic melanomas (eventual AJCC stage III/IV). Horizontal bars represent mean CXCL12 fluorescence per cell (mean +/- SD). Statistics acquired by students T test  $P = 0.113$  ns.

(c) Scatter graph representing the mean (4 HPF) of CXCL12 fluorescence per cell in all primary AJCC stage BRAF/NRAS wild-type or mutant melanomas. Horizontal bars represent mean CXCL12 fluorescence per cell (mean +/- SD). Statistics acquired by students T test  $*P = 0.032$ .

**Figure 4. Increased of CXCL12 in Primary Cutaneous Melanoma Epidermis Correlates with Increased Time to Metastasis**

Representative images of blue DAPI staining or Melan-A (green) and CXCL12 (red) expression depicted in the epidermis (a) adjacent to or (b) above an AJCC stage III primary melanoma tumour. Images were acquired with negative antibody control set to no detectable fluorescence for each channel. Images acquired by confocal microscopy with a magnification 20x scale bar = 150µm.

(c) Scatter graph representing mean (2 HPF) CXCL12 fluorescence per cell in the adjacent epidermis of eventual AJCC stage III/IV melanomas versus time to metastasis (months) after 7 year follow up. Statistics acquired by Pearsons correlation  $*P = 0.013$ .

(d) Scatter graph representing mean (2 HPF) CXCL12 fluorescence per cell in the adjacent epidermis of all AJCC stage melanomas versus relative tumoural CXCR4 expression. Statistics acquired by Pearsons correlation  $**P = 0.009$ .



**Figure 5. Expression of CXCR7 in Cutaneous Melanoma and the Primary Tumour Microenvironment**

(a) Representative images from 3 replicate experiments for the immunofluorescent expression of CXCR7 in EA.hy926 endothelial cells (positive control), metastatic melanoma cells CHL-1 or WM-164, and primary melanocytes. Images were acquired with negative antibody control set to no detectable fluorescence. Green depicts CXCR7 positivity and blue DAPI nuclear staining. Images acquired by confocal microscopy with a magnification 20x scale bar = 50µm.

(b) Representative images of CXCR7 immunohistochemical expression in a cohort of primary cutaneous melanomas of differing AJCC stage. Images depict negative CXCR7 expression in the tumour bulk of an eventual stage III primary melanoma, and positive CXCR7 expression in capillary and blood vessels within the tumour microenvironment, or following staining with an isotype negative primary antibody control. Images were acquired at x20 magnification. Arrow depicts positive CXCR7 staining in capillaries and larger blood vessels. Scale bar = 50µM.

Figure 1

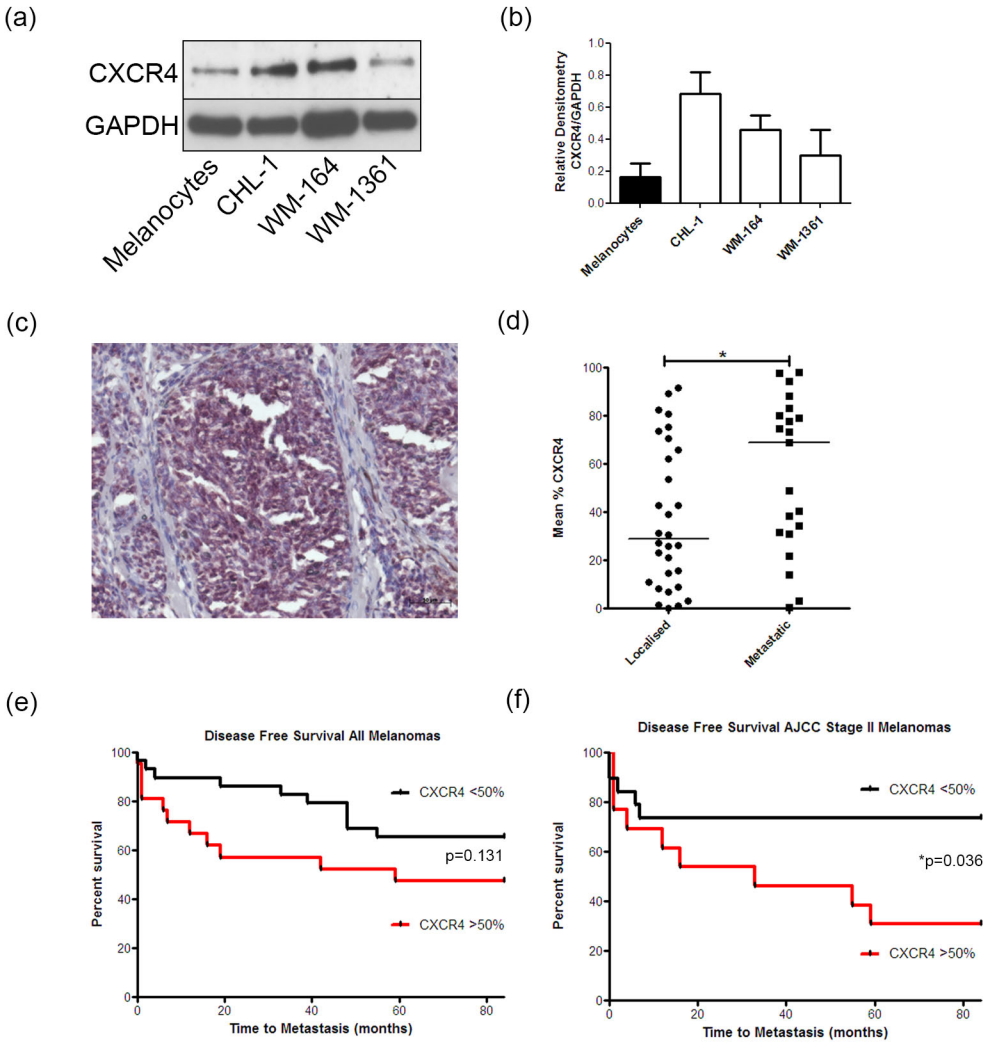


Figure 2

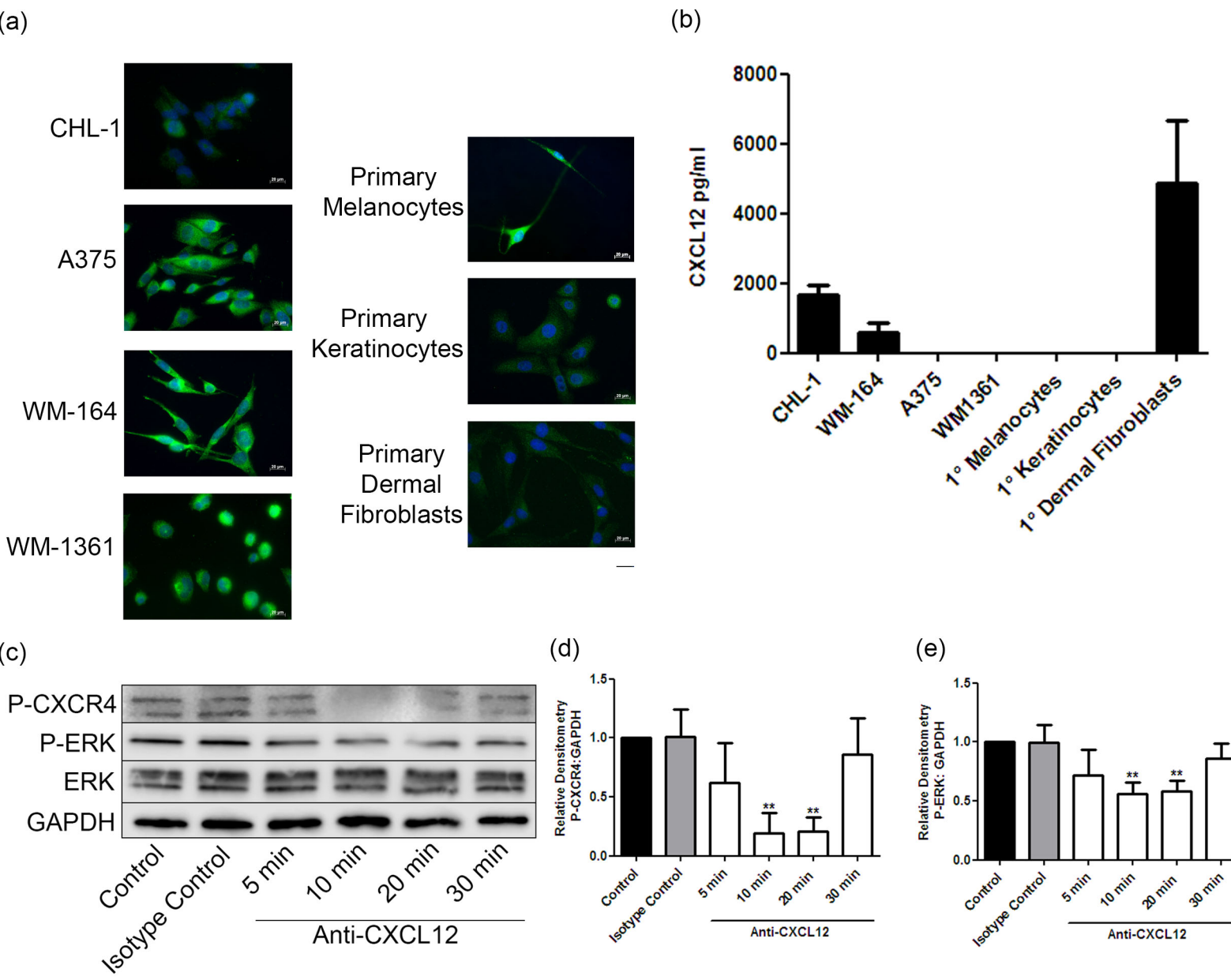


Figure 3

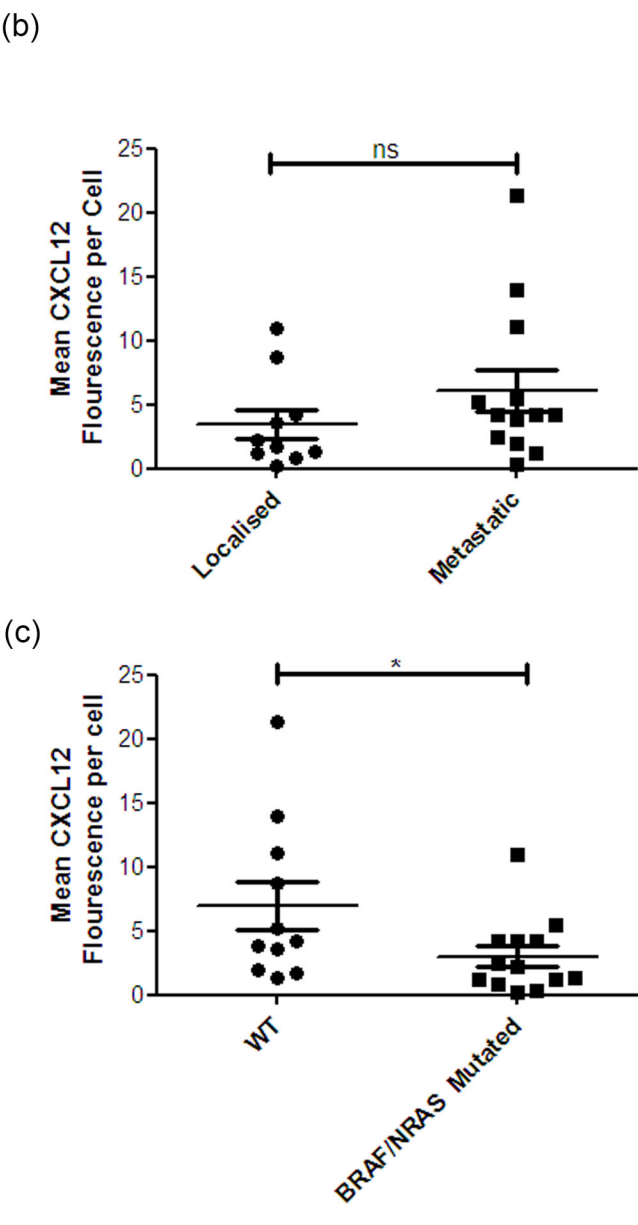
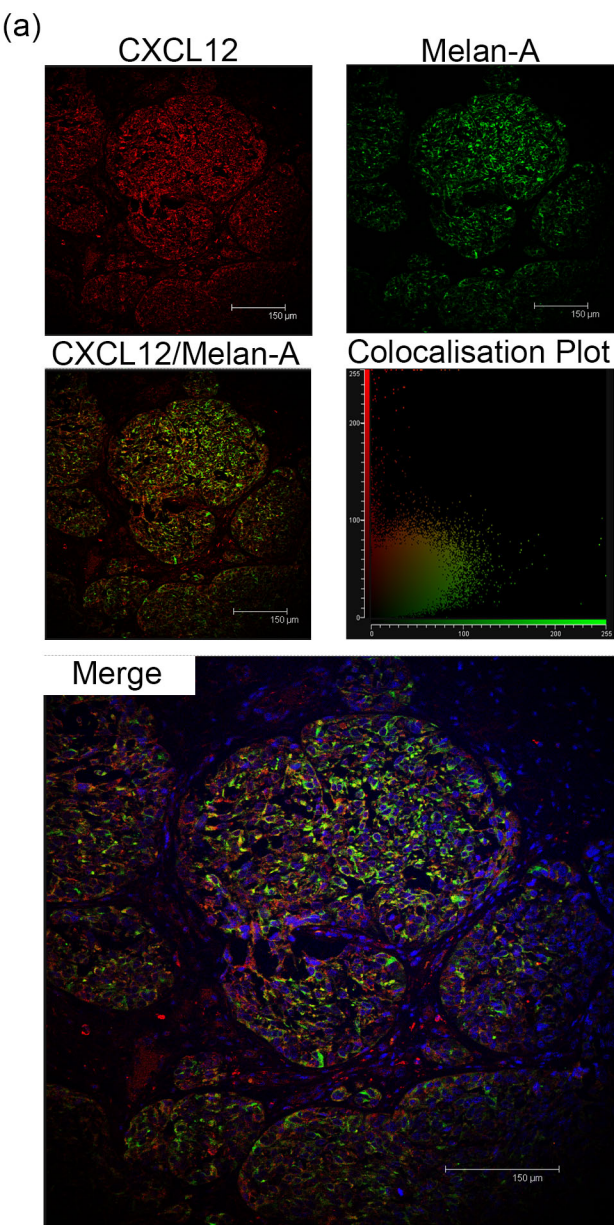


Figure 4

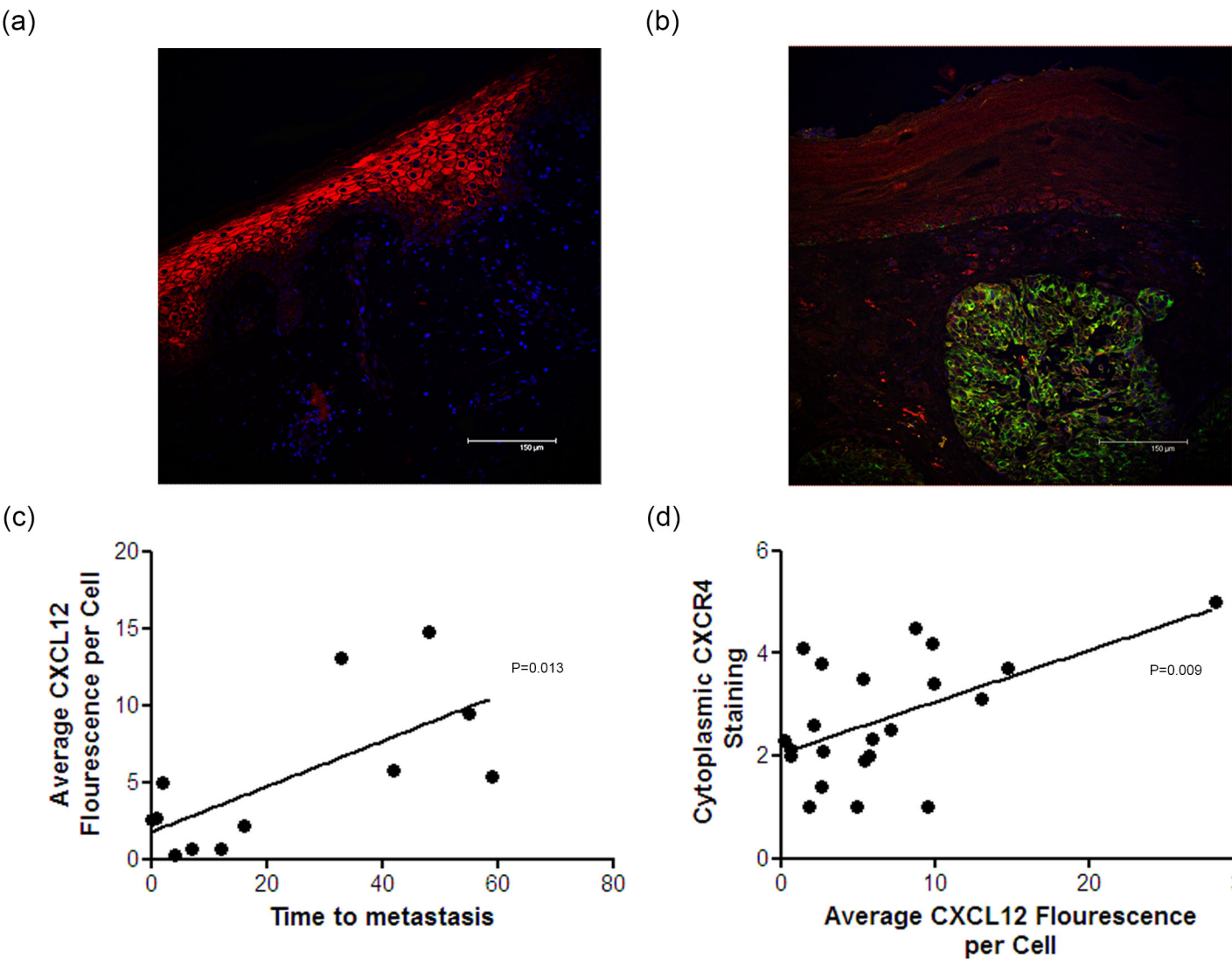
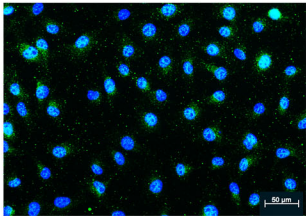




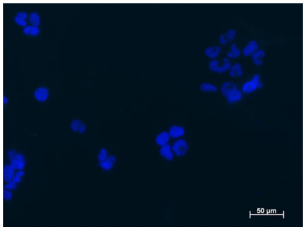
Figure 5

(a)

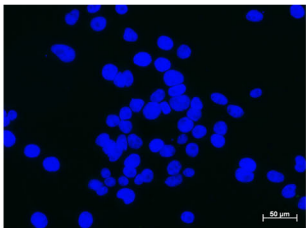
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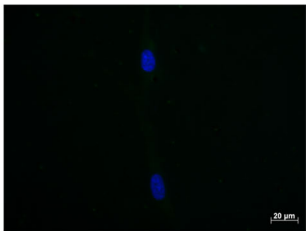
CHL-1



WM-164

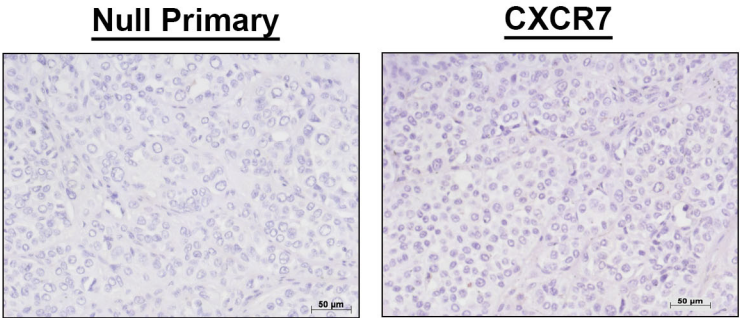


Primary  
Melanocytes

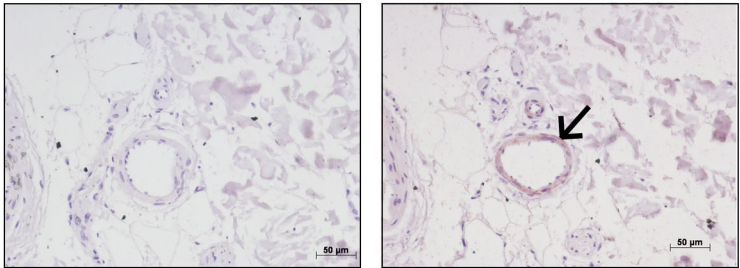


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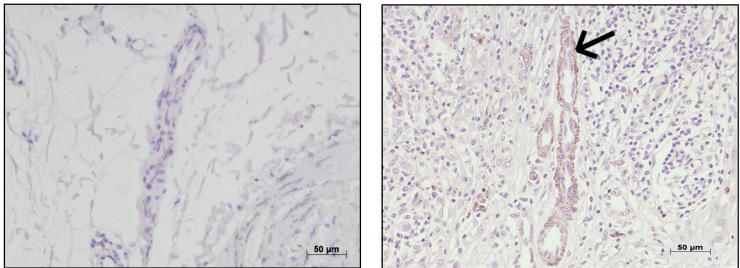
Tumour  
Stage III



Capillary



Capillary



Blood  
Vessel

